Occurrence of hemotrophic mycoplasmas in horses with correlation to hematological findings

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Occurrence of hemotrophic mycoplasmas in horses with correlation to hematological findings

Hemotrophic *Mycoplasma* infection in horses

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Abstract

Hemotrophic mycoplasmas (HM) are small, cell wall-less bacteria and infections are known for a wide range of animals. One possible indication of equine HM infection was given in 1978, when a 'haemobartonellosis' outbreak was diagnosed in Nigerian horses by microscopy. However the first molecular proof of HM in horses was not reported until 2010, when a fragment of about 900 bp of the 16S rRNA of the equine HM was obtained. This sequence was used for development of a SYBR green I real-time PCR assay specific for equine HM. The lower detection limit of the PCR was ten 16S rDNA copy numbers per ml of blood. The newly designed assay was successfully applied for the detection and quantification of HM in horses in Germany. A high sample prevalence of 26.5% (95 % CI: 18.8-35.5%) was found (31 out of 117 horses). The mean bacterial load was 1.10×10^6 16S rDNA copy number/ ml blood (range: minimum 1.05×10^3, maximum 1.27×10^7). Equine HM were also detected by microscopy (Giemsa and acridine orange stained blood smears), but results do not correlate very well with PCR results, as microscopy proved rather unspecific and not sensitive. In horses younger than one year, a significant correlation between PCR positive status and anemia was found. No correlation was found in PCR-positive animals older than one year. Therefore we assume that HM infection has a higher clinical relevance in young animals.

Keywords

Hemotrophic mycoplasma, horse, anemia, real-time PCR, SYBR green, prevalence, microscopic diagnostic.

1. Introduction

Hemotrophic mycoplasmas (HM) are uniquely adapted bacteria causing epi- or intra-erythrocytic infections in their hosts (Messick, 2004; Hoelzle 2008; Groebel et al., 2009). Formerly classified within the genera *Haemobartonella* and *Eperythrozoon* (order *Rickettsiales*) HM are now classified within the taxonomic class *Mollicutes* based on phylogenetic analysis of the 16S rRNA (Neimark et al., 2001). Infections with HM in pigs, cattle and cats are well-characterized and clinically marked by an overt life-threatening hemolytic anemia or a subtle chronic anemia, ill-thrift, infertility, lethargy, depression, weight loss, growth retardation in young animals and immune suppression. In horses the current knowledge about HM infections is rather restricted. Indications of HM infections in horses based on microscopic findings were reported in 1978 in Nigeria (Grettilat, 1978), and, recently, we found the first molecular proof of a novel HM isolate infecting horses. (Dieckmann et al., 2010). However, further studies are necessary to investigate the prevalence and significance of HM infections in horses. Since HM have never been cultivated so far, HM diagnosis relies mainly on microscopic and PCR techniques (Messick, 2004; Hoelzle, 2008). In the last years quantitative real-time PCR assays for the specific detection of several HM species have been established. (Hoelzle et al., 2007; Willi et al., 2009; Tasker et al., 2010).

Aims of this study were the determination of occurrence of HM infections in horses, and evaluation of its clinical importance with regard to the induction of anemia. Therefore an equine HM specific quantitative SYBR green I real-time PCR assay for detection of equine HM in horse blood samples was developed.

2 Material and Methods

2.1 Samples

For establishment of the SYBR green I real-time PCR assay, DNA from blood of two HM positive horses (Dieckmann et al., 2010) and 66 HM negative horses collected from horses presented at the Clinic for Horses (Vetsuisse Faculty, University of Zurich) for unrelated purposes were included. These 66 animals had been analyzed for the presence of HM by a HM-specific universal SYBR green I real-time PCR assay (Willi et al., 2009).

To screen for equine HM infections, EDTA-anti-coagulated blood samples of 117 horses originating from one breeding farm in Northern Germany with a known HM history (Dieckmann et al., 2010) were collected. This farm takes care of about 120 horses of different ages and breeds. Young horses (9 months to 2 years) were grouped according to their gender and age. During summer young horses are grazed in the marshlands of the river Weser. From October to April young horses are kept at the farm in barns with daily turn-out on pasture. Riding hoses and brood mares stay at the farm throughout the year. Blood samples were collected from 117 horses, thereof were: 70 mares (59.8%; 19 of them were pregnant (27.1%)), 17 geldings (14.5%) and 30 stallions (25.6%). Horses were of the age of 9 months to 26 years, mean age of horses was 4.3 years. 112 of the horses were warm blood breeds (95.7%); also there were two German riding ponies (1.7%), one tinker (0.9%), one thoroughbred (0.9%) and one pinto (0.9%) included into the study. Seven horses (6.0%) were reported with the following clinical preliminary report: poor nutritional condition, shaggy fur, poor performance. All horses were negative for *Theileria,*spp. *Babesia* spp., and *Bartonella* spp. infections by microscopy or culture, respectively.

2.2 Laboratory examination of horse blood samples

Hematological parameters, i.e. hematocrit (Ht), hemoglobin (Hb), red blood cell count (RBCC), white blood cell count (WBCC), mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC), were determined (synlab.vet, Geesthacht, Germany) within 24 hours after sampling. Hematological parameters were analyzed using reference values (Cowell and Tyler, 2001; Merck Veterinary Manual; http://www.merckvetmanual.com/mvm/index.jsp?cfile=htm/bc/ref_00.htm). Hematocrit was used as parameter of anemia (Ht < 0.32 l/l indicates anemia, reference range: 0.32-0.48 l/l). Giemsa and acridine orange stained peripheral blood smears were prepared.

2.3 DNA preparation

Genomic DNA was extracted from 800 µl EDTA-anti-coagulated blood by a direct lysis method (Hoelzle et al., 2003). Pellets were then used to extract genomic DNA with the GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland). Genomic DNA was eluted in 200 µl elution buffer and stored at -20°C until use. To monitor cross-contamination extraction controls consisting of PBS were performed accordingly within each batch of 12 samples.

2.4 Standard DNA

Plasmid DNA containing the partial 16S rRNA sequence of the equine HM isolate 30/7, (accession number FN421445) was used as standard DNA for determination of sensitivity of the assay and for quantification of HM (Dieckmann et al., 2010). Cloning was performed using the TOPO TA pCR2.1 cloning kit (Invitrogen, Basel, Switzerland), and the 30/7 plasmid DNA was purified using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Purity and concentration of the plasmid were checked by agarose gel electrophoresis and optical density measurements (Biophotometer,

Vaudaux-Eppendorf). Plasmid size of pCR30/7 of 4.8 kb was used to calculate the concentrations in plasmid copies per micro liter corresponding to 16S rDNA copy numbers (CN) of the equine HM isolate. Dilutions of 10^6, 10^5, 10^4 CN per reaction of the purified plasmid DNA were included as standard controls for HM quantification in each SYBR green I PCR run. Quantitative interpretation of the PCR results was obtained by the LightCycler™ software (Roche Diagnostics, Rotkreuz, Switzerland). The number of bacterial cells in horse blood samples was calculated as follows: 16S rDNA copy number per ml blood = average 16S rDNA copy numbers (determined by LC software) x 50 (Hoelzle et al., 2007).

2.5 SYBR green I real-time PCR assay

Primers were designed for amplification of a 107 bp product of a 16S rRNA region specific for the equine HM isolate (Dieckmann et al., 2010) using the ARB probe design tool (Ludwig et al., 2004). Therefore 16S rRNA sequences of hemotrophic mycoplasmas, non-hemotrophic mycoplasmas and non-mycoplasmal organisms were integrated into an ARB database, aligned using the ClustalW tool and the alignment was refined manually. At least two mismatches in the forward or reverse primer sequence were found when aligned with the 16S rRNA of nearly all HM species and other bacteria. However, no mismatches were found with the 16S rRNA of the bovine species '*Candidatus* M. haemobovis', as it is nearly identical to the 16S rRNA of the equine HM. Accession numbers, primer sequences and part of the alignment are shown in Table 1. Primer binding specificities were tested using pDRAW32 (www.acaclone.com; Tippmann, 2004) and primer sequences were searched for sequence homologies using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 1997). Primers were purchased from Eurofins MWG Operon (Ebersberg, Germany).

The LightCycler™ 2.0 System (Roche) was used in combination with the LightCycler® FastStart DNA Master^{PLUS} SYBR green I master mix kit (Roche). Each glass capillary

136 contained 15 µl of master mix (10 µl water, 1 µl primer mix (0.25 µM each), 4 µl premixed
137 master mix (5x)) and 5 µl of extracted template DNA. Cycling conditions were as follows:
138 preincubation (15 min at 95°C) and 45 cycles of amplification (10 sec 95°C, 5 sec 55°C,
139 20 sec 72°C). A melting curve was generated using the following settings: heating from 65°C
140 to 95°C with a ramp rate of 0.1°C per sec. A no template control (water) was included in each
141 run to check for contamination.
142 The assay was evaluated as positive, if two criteria were fulfilled: first, an exponential
143 increase in fluorescence acquisition during the first 32 cycles of PCR (CP < 32). Samples with
144 crossing points of 32 to 35 cycles were repeated to verify results. If the CP values were
145 consistently lower than 35 cycles, the sample would be regarded as positive. Second, the
146 melting temperature (T_M) curve showed a distinct peak at $T_M = 81 \pm 1$°C. If the curve was not
147 distinct and/ or a certain T_M determination was not possible, the samples were repeated to
148 verify results. If no amplification occurred, the CP values were > 35 cycles or no distinct T_M
149 curve could be obtained, the PCR reaction was assigned as negative. In all runs the positive
150 controls (pCR30/7 pCR plasmid DNA) were positive and the no template controls (water)
151 were negative. Only PCR runs fulfilling these criteria were taken into consideration.
152
153 *2.6 Determination of specificity and sensitivity of the new designed SYBR green I real-*
154 *time PCR assay*
155 Specificity of the assay was tested using DNA from equine HM isolates (Dieckmann et al.,
156 2010) and HM negative horse samples (n = 66). Other bacterial DNA samples used for
157 specificity testing are listed in Table 2.
158 The analytical lower limit of detection (LOD) of the SYBR green I PCR assay was
159 determined. Therefore pCR30/7 plasmid DNA was spectrophotometrically quantified and
160 DNA concentrations were adjusted to 53 pg representing 10^7 CN. Ten-fold serial dilutions

were analyzed in triplicate. The pCR30/7 plasmid DNA was calculated as 5.3 ag (attogram) per copy (genome weight = genomic length (bp) x 665 Da/bp x 1.67 x 10^{-24} g/Da (Hoelzle et al., 2007), size of pCR30/7 plasmid = 4.8 kb).

2.7 Determination of intra- and inter-assay variations

Evaluation of the reproducibility of the crossing point (CP) values and melting temperatures (T_M) were performed by analysis of the coefficient of variation (CV). For testing the intra-assay variation, three standard series of pCR30/7 plasmid DNA (10^7 to 10^1 CN) were tested within one run. Inter-assay variations were determined by performing three standard curves of pCR30/7 plasmid DNA (10^7 to 10^1 CN) on different days.

2.8 Gel electrophoresis, DNA sequencing and sequence analysis

To verify the amplicons the SYBR green I real-time PCR were completely applied on a 2% agarose gel. PCR products were excised from the gel, purified using the QIAquick Gel Extraction Kit (Qiagen AG, Hombrechtikon, Switzerland), cloned (TOPO TA Cloning Kit, Invitrogen) according to the manufacturer's instructions and sequenced (Eurofins MWG Operon). Sequences were searched for homologies using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi; Altschul et al., 2007).

2.9 Statistical analysis

Data were compiled and analyzed using Excel (Microsoft, Wallisellen, Switzerland) and R Foundation for Statistical Computing (R version 2.11.1; Vienna, Austria). For observed prevalence 95% confidence intervals (CI) were calculated. Categorical values (gender, anemia, clinical status, pregnancy, type of housing) were analyzed by Fisher's exact test and continuous variables (age, hematological parameter) by the Mann-Whitney *U*-test. Correlation

of hematocrit and blood loads was assessed by the spearman rank correlation coefficient (r_s). Differences were considered statistically as significant, if $P \leq 0.05$.

2.10 Nucleotide sequence accession numbers

The nucleotide sequences have been deposited at GenBank with accession number FR668084, FR668085 and FR668086.

3. Results

3.1 Evaluation of the SYBR green I real-time PCR assay

The SYBR green I real-time PCR assay reacted positive with DNA from equine HM isolated from horse blood. Also cross-reaction with '*Candidatus* M. haemobovis' was observed as expected. Neither other HM, nor non-HM species, nor non-mycoplasmal organisms gave a positive signal (Table 2). Additionally, all universal HM PCR negative horses (n = 66) reacted negative in the novel SYBR green I real-time PCR assay. Presence or absence and size of amplified DNA (107 bp) derived by the SYBR green I real-time PCR assay was confirmed by agarose gel electrophoresis (Fig. 1) and amplicon specificity of the positive control was verified by sequencing showing an overall identity of 100% with the primer spanned region. Sequencing of three selected PCR products revealed sequences of 105-106 bp showing an identity of 99-100% to *Mycoplasma* sp. horse isolate 30/7 (Acc. no. FN421445) and 96-98% identity to '*Candidatus* M. haemobovis' (Acc. no. EF460765).

For analysis of sensitivity ten-fold serial dilutions of pCR30/7 plasmid DNA ranging from 5.3 ag to 53 pg (corresponding to 10^0 to 10^7 CN) per reaction were tested in the SYBR green I real-time PCR assay. PCR reacted positive if at least 10 CN/ ml blood were present.

Intra- and inter-assay variations were measured to determine the reproducibility of the SYBR green I real-time PCR assay. The coefficients of variation of CP and T_M values for the intra- and inter-assay variations are presented in Table 3.

3.2 Evaluation of significance of HM infection in horses by SYBR green I real-time PCR

To evaluate the significance of HM infection in horses, 117 blood samples of anemic and non-anemic horses were tested. Thirty-one horse blood samples (26.5%; 95% CI: 18.8-35.5%) revealed a positive SYBR green I real-time PCR result showing a mean crossing point of CP = 32.28 ± 1.44 (range: minimum 29.43, maximum 34.69) and a mean melting temperature

of T_M = 80.88 ± 0.37 °C (range: minimum 80.16, maximum 81.78). Nine horses showing an anemia (Ht < 0.32; 9/23, 39.%), ten horses with a low level hematocrit (Ht = 0.32-0.34; 10/38, 26.3%) and twelve non-anemic horses (Ht > 0.34; 12/56, 21.4%) exhibited positive PCR results.

All equine blood samples that tested positive by the newly designed SYBR green I real-time PCR assay were further analyzed for HM quantification. The overall mean bacterial load was 1.10 x 10^6 16S rDNA copy number/ ml blood (range: minimum 1.05 x 10^3, maximum 1.27 x 10^7). In anemic and PCR positive horses a mean bacterial load of 1.10 x 10^6 16S rDNA copy number/ ml blood (range: minimum 1.28 x 10^3, maximum 4.15 x 10^6) was observed and in non-anemic and PCR positive horses a mean bacterial load of 1.42 x10^5 16S rDNA copy number/ ml blood (range: minimum 1.05 x 10^3, maximum 1.27 x 10^7) was detected. In the group of horses (n = 7) with the preliminary report of showing a reduced performance condition, having a shaggy fur and being meager, two horses showed a positive PCR signal (28.6%; 95% CI: 3.7-71.0%) with a mean bacterial load of 4.65 x 10^3 16S rDNA copy number/ ml blood (range: minimum 1.05 x 10^3, maximum 8.25 x 10^3).

3.3 Hematological findings

Ht, Hb, RBCC, WBCC, MCV, MCH and MCHC were analyzed and compared to reference values (Table 4). Twenty-three of 117 horses (28.2%) were anemic (Ht < 0.32), whereas 38 more horses (32.5%) showed a low level Ht within the reference range (0.32-0.34 l/l). Mean Ht was 0.34 l/l. Mainly young horses (< 1 year) were affected from low Ht values. They mostly showed a low level Hb concentration and a low level RBCC, too. Mean Hb of all animals (n = 117) was 128 g/l and mean RBCC was 8.80 x 10^{12} cells/l. Some horses exhibited a leucocytosis. The overall (n = 117) mean WBCC was 9.6 x 10^9 cells/l. Mean MCV was 39.47 fl, mean MCH 14.67 pg and mean MCHC 37.26 g/dl (Table 4).

243

244 *3.4 Characteristics of HM PCR-positive horses*

245 Looking at the complete population investigated, PCR-positive horses had significantly lower
246 RBCC and MCHC than PCR-negative horses ($P \leq 0.05$; Table 4; Fig. 3). In addition, Ht and
247 Hb tended to be lower in PCR-positive horses compared with PCR-negative animals (Table
248 4). No significant difference was found in WBCC, MCV and MCH. However, in horses
249 < 1 year, Ht, Hb, and RBCC were significantly lower in PCR-positive compared with PCR-
250 negative horses ($P \leq 0.05$; Table 4; Fig. 3), whereas MCV and MCH were significantly
251 elevated in PCR-positive horses < 1 year ($P \leq 0.05$; Table 4). In contrast, in PCR-positive
252 animals > 1 year all measured blood parameters were not significantly different compared
253 with PCR-negative horses. PCR-positive horses < 1 year showed significantly lower Ht and
254 Hb values ($P < 0.0001$) as well as lowered MCV and MCH than PCR-positive horses > 1 year
255 ($P = 0.0005$ and $P < 0.0001$, respectively) and a significantly higher WBCC ($P \leq 0.05$), but
256 not a significantly changed RBCC (Fig. 4). No significant correlations between bacterial
257 blood loads and blood parameters could be found (assessed by the spearman rank correlation
258 coefficient r_s). PCR-positive status of the overall population independent from age was not
259 significantly associated with anemia. However, in horses < 1 year anemia was associated with
260 infection. In general, PCR-positive and PCR-negative horses showed no significant
261 differences in age, gender, pregnancy and clinical health status.

262

263 *3.5 Comparison of SYBR green I real-time PCR results to microscopic detection methods*

264 Results of SYBR green I real-time PCR assay were compared to results of microscopy of
265 Giemsa and acridine orange stained peripheral blood smears (Fig. 2). Forty-six of 117
266 samples (39.3%) revealed HM in peripheral blood smears stained with either Giemsa or
267 acridine orange. In 17 Giemsa (14.5%) and in 44 acridine orange (37.6%) stained horse blood

268 smears HM were detected as coccoid structures of approx. 0.3-0.4 µm in size closely attached
269 to the surface of RBCs. In fifteen samples (12.8%) HM could be detected in both staining
270 techniques and 71 samples (60.7%) were negative in both staining techniques.
271 Twenty-five of 46 samples microscopically diagnosed as HM positive (either Giemsa or
272 acridine orange stained) were confirmed as positive by PCR (54.3%). In 71 microscopically
273 HM-negative samples, six reacted positive in PCR (8.5%). This corresponds to a rate of false-
274 positive microscopic results of 23.3% and false-negative microscopic results of 19.4%.
275

276 **4. Discussion**

277 Although the first possible evidence of equine HM infections was described a long time ago
278 by microscopy (Grettilat, 1978) our knowledge of HM in horses is rather limited. Recently the
279 molecular confirmation of HM in horses was published for the first time (Dieckmann et al.,
280 2010).
281 In this study we describe the development of the first SYBR green I real-time PCR assay for
282 the sensitive and specific detection of HM in the blood of horses and the application of the
283 assay for the detection of HM in a horse population in Germany and analysis of its clinical
284 importance. Formerly the diagnosis of HM infections was based on the microscopic detection
285 in chemically stained blood smears – a method which is not specific and sensitive enough to
286 detect animals with low bacterial blood loads (Hoelzle, 2008; Ritzmann et al., 2009). For
287 other HM species, e.g. *M. haemofelis*, *M. suis*, *M. haemocanis* and '*Candidatus*
288 M. turicensis', the establishment of specific and sensitive real-time PCR methods has
289 delivered valuable insights into the significance of HM infections in cats, dogs and pigs
290 (Hoelzle et al., 2007; Barker et al., 2010; Willi et al., 2009). Accordingly the reliable
291 quantitative detection of HM infections in horses is the crucial step for the evaluation of the
292 actual significance of equine HM. Our newly developed SYBR green I real-time PCR assay

293 demonstrated a sensitivity of 10 CN/ ml blood. The analytical specificity of the SYBR green I
294 real-time PCR was confirmed by testing DNA from different bacteria (mycoplasmas and
295 other bacteria with clinical relatedness). As predicted *in silico* only cross reactivity was found
296 with the '*Candidatus* M. haemobovis' DNA.
297 Comparison of the PCR and microscopic results revealed a poor correlation. To some extent
298 microscopically positive tested animals reacted negative in PCR and vice versa. False-
299 negative microscopic detection may result from low bacterial loads or inapparent infections
300 with bacteria being absent from blood, whereas false-positive microscopic results are because
301 of confusion of HM with Howell-Jolly or Heinz bodies, background debris and staining
302 artifacts (Hoelzle 2008). It was reported earlier that blood loads of 4.4×10^6 '*Candidatus*
303 M. turicensis' copies/ ml blood correspond to only one bacterium per 10^3 to 10^4 RBCs
304 (Museux et al., 2009). Bacterial blood loads of affected horses in this study were in the same
305 range hindering the unambiguous identification of HM on the surface of RBCs by
306 microscopy. Using the newly designed SYBR green I real-time PCR assay an unambiguous
307 and sensitive detection of HM in blood of affected horses was possible. The possibility of
308 false-positive or false-negative SYBR green I real-time PCR results is negligible due to its
309 high specificity and sensitivity. However, it remains difficult to establish a novel diagnostics
310 tool, when a good reference method is missing. Thus, assignment of positive and negative
311 predictive values was hampered.
312 The HM prevalence in the horse population investigated in this study was 26.5% (95% CI:
313 18.8-35.5%). Analogically high HM prevalences of 22.3% and 18.6%, respectively, were
314 found in cattle without apparent clinical signs in Japan ('*Candidatus* M. haemobovis',
315 Tagawa et al., 2010) and in alpacas in Switzerland (Kaufmann et al., 2010). In comparison,
316 prevalences of feline (0.5 to 8.5%; Willi et al., 2006) and canine HM in Switzerland (1.2%;
317 Wengi et al., 2008) are considerably lower. Since our study includes horses of only one farm

318 the general prevalence and pathogenic potential of equine HM cannot be assessed on the basis
319 of the present data. Further studies are necessary to determine the actual prevalence and
320 whether the found HM infections may be a regional phenomenon or a single-herd, sporadic
321 event.
322 Younger horses seem to be more affected by an equine HM infection. Though red blood cell
323 count (RBCC) and mean cell hemoglobin concentration (MCHC) were significantly lowered
324 in all PCR-positive animals. RBCC, hematocrit (Ht), hemoglobin (Hb), mean cell volume
325 (MCV) and mean cell hemoglobin (MCH) were significantly lowered in infected animals
326 < 1 year, but not in infected horses > 1 year (Table 4). However, changes are mostly within
327 the normal range, but at a low level. As the number of samples in each group highly differs,
328 the calculated statistics should be viewed with caution. Therefore, this correlation in horses
329 <1 year old should be investigated in future studies of whether an equine HM infection has a
330 more severe course of disease in young horses, as it was described for HM infection in llamas
331 (Reagan et al., 1990) and sea lions (Volokhov et al., 2011). When interpreting changes in
332 blood parameter, one have to be aware, that blood counts of young horses may generally
333 differ from older horses (Harvey et al., 1984; Tyler et al., 1987; Cowell and Tyler, 2001) but
334 no distinct reference ranges for younger horses are given. Reference ranges can slightly vary
335 from country to country and from race to race (especially for drafting horses, thoroughbreds,
336 and ponies (Harvey et al., 1984; Tyler et al., 1987; Cowell and Tyler, 2001; Fey, 2006;).
337 Differences in blood counts of young animals may also be affected by higher possibility of
338 intestinal parasites. Overall looking at the entire population independent of age, equine HM
339 infection was not associated with anemia and blood loads do not correlate with Ht values.
340 Absence of correlation of anemia and HM infection was also reported for feline HM (Willi et
341 al., 2006) whereas *M. suis* infection in pigs is often accompanied by severe anemia and strong
342 correlation of Ht, Hb and RBCC (Hoelzle, 2008; Ritzmann et al., 2009).

In horses, HM infection most often seems to have a subclinical or asymptomatic course of disease showing only low bacterial loads in blood. For this reason detection of HM in horse blood as well as assignment of a distinct clinical manifestation is complicated. Mostly, horses only show unspecific clinical signs like slimming, shaggy fur, apathy and a bad general performance.

HM were detected by SYBR green I real-time PCR in healthy horses without any clinical signs and without showing abnormalities in the blood count. A similar situation was reported for '*Candidatus* M. haemolamae' and *M. haemocanis*. Disease and anemia seem to be developed only by stressed, immunocompromised or co-infected animals (Messick, 2004 Tornquist et al., 2009). For *M. wenyonii* it was described that clinical signs were more present in combination with concurrent infections with *Anaplasma marginale* (Hofmann-Lehmann et al., 2004). An undulating course of infection alternating between pronounced clinical signs and an asymptomatic stage was described for feline HM and *M. suis* (Hoelzle, 2008; Willi et al., 2006).

Interestingly, infections of cattle herds with a rather similar HM species ('*Candidatus* M. haemobovis') were reported in the same region (Hoelzle et al., 2011). Both, equine and bovine HM isolates, exhibits high 16S rRNA sequence identity (97-98%). According to the cut-off values (Drancourt and Raoult, 2005) this sequence difference may be not enough for designation of a novel species. Therefore it could be speculated that the equine HM isolate and '*Candidatus* M. haemobovis' could be one species cross-infecting cattle and horses. Host specificity of mycoplasmas was recently questioned (Pitcher and Nicholas, 2005). In respect of historical reasons HM species were named after the animal species they were isolated from. A similar situation as in horse and cattle is given with *M. haemofelis* (cat) and *M. haemocanis* (dog), which shows a 16S rRNA sequence identity of 99.3-99.7%, but only 94.3-95.5% sequence identity of the RNase P (*rnpB*) gene (Peters et al., 2008; Sykes et al., 2005). Further

phylogenetic analyses (e.g. sequencing of the *rnpB* gene) and cross-infectivity studies are necessary to determine if the novel equine HM isolate is an independent species with the proposed designation '*Candidatus* Mycoplasma equi'. Unfortunately amplification and sequencing of the full length 16S rRNA and the *rnpB* gene failed during this study as well as in our previous study (Dieckmann et al., 2010).

In conclusion we confirmed HM infections in Northern Germany using our newly designed SYBR green I real-time PCR assay. The clinical significance of equine HM infections still remains difficult to interpret and only in horses younger than one year equine HM infection was associated with anemia. More investigations in the field of clinical characterization of this novel infection in horses have to be done in future to establish a clear clinical picture and to examine the pathogenic potential of HM infection in horses. The herein developed SYBR green I real-time PCR assay provides an important prerequisite for doing so.

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497

498 **Figure captions**

499 **Fig. 1.** SYBR green I real-time PCR assay of 10 samples applied on an 2% agarose gel. An
500 amplicon of the corresponding size of 107 bp was obtained in PCR positive horses
501 (lane 3 = no. 90; 4 = no. 103; 5 = no. 108; 6 = no. 111; 7 = no. 114; 9 = no. 126; 10 = no. 127)
502 and amplification was absent in no template control (lane no. 1) and PCR negative horses
503 (lane 2 = no.86; lane 8 = no. 117). 100 bp ladder (lane 11) was used for accession of amplicon
504 sizes.

505

506 **Fig. 2.** Micrographs of Giemsa (A) and acridine orange (B) stained peripheral blood smears
507 (magnification 1000x) of a horse infected with hemotrophic mycoplasmas. Roundish particles
508 of approx. 0.3-0.4 µm in size are detected on the surface of red blood cells in both staining
509 techniques. In Giemsa stained blood smear it can be seen, that some hemotrophic
510 mycoplasmas are laying in small grooves on the surface of the erythrocytes.

511

512 **Fig. 3.** Blood parameters ((A) hemoglobin, (B) hematocrit, (C) red blood cell count, (D) white
513 blood cell count) of PCR positive (PCR pos) and PCR negative (PCR neg) horses are
514 presented as box plots. Results are shown for all horses and for horses grouped according to
515 their age (< 1 year, > 1 year). Boxes extend from the 25^{th} to the 75^{th} percentile, median is
516 presented as horizontal line and the bars extend to the minimum and maximum value. Dots
517 represent outliers. Values from PCR-positive and PCR-negative horses were analyzed for
518 significant differences using the Mann-Whitney *U*-test. *P*-values are listed in Table 4.

519

520 **Fig. 4.** Comparison of correlation between blood parameters ((A) hemoglobin, (B)
521 hematocrit, (C) red blood cell count, (D) white blood cell count) and bacterial load in PCR

positive horses > 1 year (n = 23) and PCR positive horses < 1 year (n = 8)). Grey boxes indicate the corresponding reference ranges according to Cowell and Tyler (7).

Table 1. Alignment of primer sequences with target and non target species 16S rRNA sequences of closely related organisms

Species	Forward primer 5'-3'	Reverse primer 5'-3'	Acc. No.
Primer sequence	CAGGCGGATGTGTAAGTTC	CGCCTCCGGTGTTCTTAAAC	
Novel equine HM isolate 30/7	-------------------	--------------------	FN421445
'*Candidatus* Mycoplasma haemobovis'	-------------------	--------------------	EU367965
Mycoplasma haemofelis	-------------------	------T----------G-	AY150984
'*Candidatus* Mycoplasma haemominutum'	T-------CAAA-TGA-CT	A-----T-------C-C--T	DQ157149
'*Candidatus* Mycoplasma turicensis'	----------AA-------	------T-----------T	DQ825450
Mycoplasma haemomuris	---------TG--------	------T---------G--T	U82963
Mycoplasma coccoides	----------AAC------	------T-----------T	AY171918
Mycoplasma suis	T----T--A----GTA-C-	A-----T-------C-C--T	U88565
Mycoplasma wenyonii	T------GGAG--TGA-C-	A-----T-------C-C--T	AF016546
Mycoplasma ovis	T------GGAG-CTGA-C-	A-----T-------C-C--T	AF338268
'*Candidatus* Mycoplasma haematoparvum'	T-------CAA--TGA-GT	A-----T-------C-CCA-	AY532390
'*Candidatus* Mycoplasma haemodidelphis'	T-------CAGTCTGA-C-	-----TT-------C-C--T	AF178676
'*Candidatus* Mycoplasma kahanei'	T-------GAGT-TGA-CT	A-----T-------C-C--T	AF338269
'*Candidatus* Mycoplasma haemolamae'	T---T---G-A--TGA-C-	A-----T-------C-C--T	AF306346
Mycoplasma pneumoniae	---------TGAA----CT	----A-T-------C-TC-T	NC_000912
Mycoplasma arginini	T----T-T-TAT-----CT	-----TT---------CC-T	M24579
Mycoplasma penetrans	---------T-AC----CT	------T---------CC-T	NC_004432
Escherichia coli	-------T-TGT-----CA	----A-----A---C-CC-G	U18997
Bacillus cereus	----T--T-TCT-----CT	----A-T-------C-CC-T	AY138272
Bacillus subtilis	-------T-TCT-----CT	----A-T-------C-CC-T	AB042061
Salmonella enterica	-------T-TGT-----CA	----A-----A---C-CC-G	U92197
Bartonella quintana	T--------ATT-----CA	----A-T-------C-CCGA	BX897700
Streptococcus equi	-------T-TGA-----CT	----A---------C-CC-T	DQ303186
Clostridium perfringens	T---------AT-----GG	----A-T---------CCTA	AB045286

Only bases different to primer sequences are shown

Table 2. Bacterial strains used for evaluation of specificity of the newly designed SYBR green I real-time PCR assay

Species	Origin	Result
Horse isolate No. 30/7	Field strain[a]	Positive
Horse isolates	Field samples, n = 117	31 positive
Horse blood controls	Field samples[b], n = 66	Negative
'*Candidatus* Mycoplasma haemobovis'	Field strains[c], n = 6	Positive
Mycoplasma haemofelis	[d]	Negative
Mycoplasma haemominutum	[d]	Negative
'*Candidatus* Mycoplasma turicensis'	[d]	Negative
Mycoplasma wenyonii	Field strains[c], n = 4	Negative
Mycoplasma suis	Strain 3808[e]	Negative
Mycoplasma suis	Strain 3806[e]	Negative
Mycoplasma suis	Strain 146[e]	Negative
'*Candidatus* Mycoplasma haemolamae'	Field strains[f], n = 5	Negative
Mycoplasma arginini	[g]	Negative
Mycoplasma penetrans	[g]	Negative
Mycoplasma pneumoniae	Sebastian Schmidl[h]	Negative
Bacillus cereus	isolated from horse[g]	Negative
Bartonella bacilliformis	Christoph Dehio[i]	Negative
Bartonella henselae	Christoph Dehio[i]	Negative
Bartonella quintana 'Fuller'	Christoph Dehio[i]	Negative
Bartonella quintana 'Toulouse'	Christoph Dehio[i]	Negative
Bartonella tribocorum	Christoph Dehio[i]	Negative
Clostridium perfringens Type A	isolated from horse[g]	Negative
Escherichia coli	isolated from horse[g]	Negative
Salmonella sp.	isolated from horse[g]	Negative
Streptococcus equi	isolated from horse[g]	Negative

[a] Field strain isolated from an anemic horse (Dieckmann et al., 2010), used as positive control for the SYBR green I real-time PCR assay (see material and methods); [b] DNA of horse blood samples collected from horses presented at the Clinic for Horses, Equine Department, Vetsuisse Faculty, University of Zurich, for unrelated purposes; negative for

any HM infection (SYBR green I real-time PCR assay covering any HM species (Willi et al., 2009)); [c] Field strains isolated from anemic cattle (Hoelzle et al., 2011); [d] DNA of three feline HM isolates from anemic cats (Willi et al., 2006); [e] Blood from splenectomized pigs experimentally infected with *M. suis* (Felder et al., 2012); [f] Field strains isolated from anemic alpacas (Institute of Veterinary Bacteriology, University Zurich); [g] Strain collection of Institute of Veterinary Bacteriology; [h] provided by Sebastian Schmidl (Department of General Microbiology, Georg-August-University of Göttingen); [i] DNA provided by Christoph Dehio (Infection Biology, Biozentrum, University of Basel).

Table 3. Inter-assay and intra-assay reproducibility of the newly designed SYBR green I real-time PCR assay

	Inter-assay variation				Intra-assay variation			
Copy number[a]	**Mean CP (± SD)[b]**	**CV (%)[c]**	**Mean T_M (± SD)[b]**	**CV (%)**	**Mean CP (± SD)[b]**	**CV (%)**	**Mean T_M (± SD)[b]**	**CV (%)**
10^7 CN[d]	22.46 ± 0.032	0.143	81.19 ± 0.092	0.126	22.34 ± 0.097	0.435	81.32 ± 0.031	0.038
10^6 CN	26.32 ± 0.233	0.885	81.17 ± 0.076	0.093	26.21 ± 0.010	0.038	81.26 ± 0.030	0.037
10^5 CN	28.89 ± 0.586	2.029	81.19± 0.025	0.031	29.10 ± 0.127	0.435	81.18 ± 0.025	0.031
10^4 CN	26.57 ± 0.385	1.449	81.17 ± 0.040	0.050	26.36 ± 0.537	2.036	81.20 ± 0.015	0.019
10^3 CN	32.78 ± 2.874	8.766	81.26 ± 0.096	0.119	34.28 ± 0.212	0.619	81.30 ± 0.057	0.070
10^2 CN	28.24 ± 2.517	8.914	81.20 ± 0.121	0.150	28.02 ± 2.564	9.151	81.38 ± 0.038	0.047
10^1 CN	31.37 ± 3.097	9.873	81.22 ± 0.339	0.418	33.56 ± 2.120	3.211	81.46 ± 1.558	2.036

[a] Copy number were calculated from spectrophotometrically quantified DNA as described in the material and methods section; [b] Mean crossing point (CP), mean melting temperature (T_M) and standard deviations (SD) were calculated from triplicates of each concentration; [c] coefficient of variation; [d] 16S rDNA copy numbers (CN).

15 **Table 4.** Hematological values of PCR-positive and PCR-negative horses

	PCR positive (n = 31)		PCR positive (n = 31)			
Parameter	**Median**	**95% CI[a]**	**Median**	**95% CI**	**Reference range[b]**	***P***
Ht[c]	0.35	0.32-0.37	0.34	0.31-0.36	0.32-0.48 l/l	0.133
Hb[d]	129.50	120.25-137.75	124.00	115.50-132.50	100-180 g/l	0.069
RBCC[e]	8.89	8.39-9.52	8.42	7.92-8.97	6.0-12.0 x 10^{12} cells/l	0.001*
WBCC[f]	9.80	7.93-11.18	9.20	7.45-11.10	6.0-12.0 x 10^{9} cells/l	0.700
MCV[g]	38.20	35.23-42.98	38.80	36.20-43.90	34-58 fl	0.187
MCH[h]	14.65	13.23-15.80	14.70	13.60-16.30	13-19 pg	0.301
MCHC[i]	37.50	36.60-38.18	37.00	36.95-37.80	31-37 g/dl	0.029*
	PCR negative, > 1 year (n = 64)		**PCR positive, > 1 year (n = 23)**			
Parameter	**Median**	**95% CI**	**Median**	**95% CI**	**Reference range**	***P***
Ht	0.36	0.34-0.38	0.35	0.34-0.37	0.32-0.48 l/l	0.196
Hb	133.00	126.00-140.00	129.00	124.00-135.50	100-180 g/l	0.105
RBCC	8.81	8.29-9.25	8.42	7.95-8.94	6.0-12.0 x 10^{12} cells/l	0.094
WBCC	9.30	7.58-10.93	8.30	7.35-10.75	6.0-12.0 x 10^{9} cells/l	0.652
MCV	40.90	37.18-43.95	42.40	37.35-45.85	34-58 fl	0.275
MCH	15.30	14.20-15.95	15.10	14.15-16.60	13-19 pg	0.381
MCHC	37.30	36.40-38.20	37.00	36.15-37.80	31-37 g/dl	0.069
	PCR negative, < 1 year (n = 22)		**PCR positive, < 1 year (n = 8)**			
Parameter	**Median**	**95% CI**	**Median**	**95% CI**	**Reference range**	***P***
Ht	0.31	0.30-0.33	0.30	0.29-0.31	0.32-0.48 l/l	0.023*
Hb	118.50	111.25-123.00	110.50	106.75-113.75	100-180 g/l	0.021*
RBCC	9.41	8.82-9.86	8.43	7.84-9.06	6.0-12.0 x 10^{12} cells/l	0.013*
WBCC	10.75	9.80-11.65	11.25	8.80-11.68	6.0-12.0 x 10^{9} cells/l	0.593
MCV	33.35	32.55-34.53	34.55	34.18-36.80	34-58 fl	0.021*
MCH	12.60	12.33-12.90	13.15	12.90-13.23	13-19 pg	0.015*
MCHC	37.75	37.10-38.08	37.25	36.15-38.03	31-37 g/dl	0.174

16 Results of hematological analysis are shown for all horses and for horses grouped according to their age (> 1 year; < 1 year); [a] confidence interval; [b] Reference range according to
17 Cowell and Tyler, 2001;[c] hematocrit; [d] hemoglobin; [e] red blood cell count, [f] white blood cell count; [g] mean cell volume, [h] mean cell hemoglobin, [i] mean cell hemoglobin
18 concentration, * significant if $P \leq 0.05$.